(12) EUROPEAN PATENT APPLICATION

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(54) Fluorescent labels.

(57) There are described stable fluorescent labels comprising a complex of Eu^{+3} and a chelating agent comprising a nucleus which is a triplet sensitizer having a triplet energy greater than that of Eu^{+3} and at least two heteroatom-containing groups which form coordinate complexes with Eu^{+3} and a third heteroatom-containing group or heteroatom in or appended to the triplet sensitizer which chelating agents are polypyridine or phenanthroline compounds. Labeled physio-logically active materials useful in specific binding assays such as labeled antigens, haptens, antibodies, hormones and the like comprising the stable fluores-cent labels having physiologically active materials adsorbed or bonded thereto are also described.

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FLUORESCENT LABELS

The present invention relates to novel fluorescent labels and more particularly to fluorescent labels useful for the preparation of specific binding reagents comprising fluorescent labeled physiologically active materials.

Fluorescent immunoreagents comprise at least one member of the immune system, i.e. an antibody or
5 an antigen, "conjugated" with a rare-earth chelate. Such "conjugation" can be achieved in one of two ways:

1) by labeling, i.e. attaching the rare-earth chelate to the antigen as described in Fluorescent Antibody Techniques and Their Application by A. Kawamura, Ed., University Park Press, Baltimore, Maryland, 1969, and then adding antibody to the conjugated antigen whereby the antibody and antigen join in the usual fashion, or
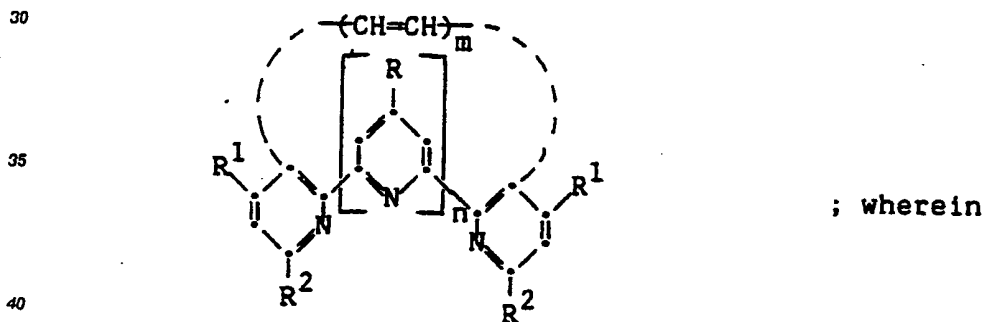
10 2) by covalent bonding of the antibody of the chelate via a chemical group which binds to both antibodies and the chelates.

U.S. Patent 4,637,988 discloses cyclic nitrogen compounds such as terpyridines and phenanthrolines as chelating agents. Such agents form chelates with lanthanide metals such as europium. The chelates can be used as fluorescent labels for physiologically active materials such as antigens and hormones in immunoas-
15 says. However, the patent only discloses one terpyridine and one phenanthroline.

Studies have shown that the specifically disclosed phenanthroline has a low quantum efficiency and is too slow in coming to fluorescence equilibrium. These performance defects make such phenanthroline impractical for use in the kind of rapid immunoassays being used today. The one specifically disclosed, terpyridine, has useful fluorescence but a higher level of fluorescence is desirable for more accurate assay
20 measurements in immunoassays.

It is the objective of the present invention to provide chelating agents having one or more improved properties such as improved quantum efficiency resulting in improved fluorescence and/or faster rates of establishing fluorescence equilibrium. That objective is achieved with chelating agents which form highly fluorescent chelates with Eu^{+3}. The chelating agent comprises a nucleus which is a triplet sensitizer having
25 a triplet energy greater than that of Eu^{+3} and at least two heteroatom containing groups which form chelates (coordinate complexes) with Eu^{+3} and a third heteroatom containing group or heteroatom which is in the sensitizer or appended to the sensitizer nucleus.

The chelating agent has the general structure

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; wherein

R represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, aryloxy, a heterocyclic group, an enzyme, an antigen or an antibody;
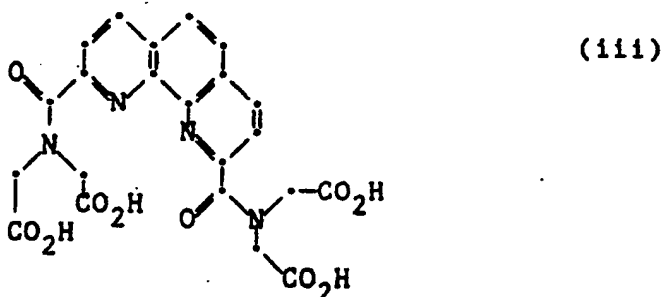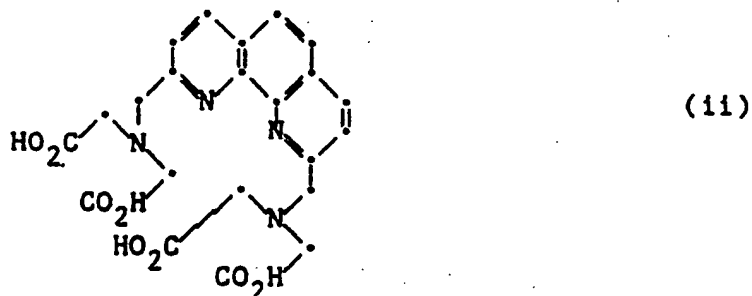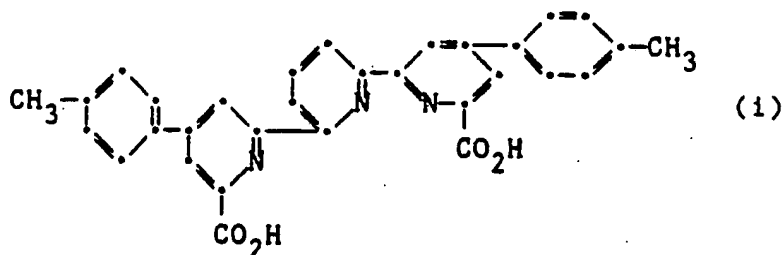
45 R^1 represents hydrogen, alkyl, alkoxy, alkyl thio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody;

R^2 represents carboxy, hydroxy carbonyliminodiacetic acid, methyleneimino diacetic acid, hydra zinylylidene diacetic acid or the esters or salts of such acids;

n is 0 to 4; and
50 m is 0 to 1

provided that a) m can be 1 only when n is 0 and b) compounds of the structures (i), (ii) and (iii) are excluded from said general structure.

(i)

(ii)

(iii)

Alkyl refers to straight or branched alkyl of 1 to 12 carbon atoms, preferably 1 to 6 such as methyl, ethyl, isopropyl, 2-ethylhexyl, decyl, etc.

The alkyl portion of alkoxy, alkythio, alkyamino has this same meaning.

Aryl and aryloxy refers to substituted or unsubstituted aryl or aryloxy of about 6 to 20 carbon atoms such as phenyl, naphthyl and phenanthryl including substituted derivatives thereof such as nitrophenyl, hydroxyphenyl, tolyl, xylyl, methoxy phenyl, methylthiophenyl, carboxyphenyl, 5,5-di-phenyl-2,4-imidazolidinedione-3-ylmethylphenyl (a diphenylhydantoin substituent on a tolyl group) and the like.

Heterocyclic refers to substituted or unsubstituted heterocyclic groups having 5 to 6 nuclear carbon and hetero atoms such as pyridyl, methylpyridyl, nitropyridyl, methoxypyridyl, oxazolyl, imidazolyl, pyrazolyl, quinolyl, etc.

The alkyl, alkoxy, alkythio, alkyamino, aryl, aryloxy, and heterocyclic groups can be part of, or have appended thereto, proteins for use in biological assays, particularly haptens, enzymes, antibodies, and antigens for example the previously mentioned hydantoin substituent.

The chelates are water-soluble, stable at low concentrations at pH of 7 to 10, highly sensitive, and have favorable molar extinction coefficients (10,000-40,000) and favorable λmax. Accordingly, the present invention provides a class of highly efficient, aqueous stabilized fluorescent labels for physiologically active materials such as antigens, hormones, antibodies and enzymes.

In general, the chelating agents exhibit improved fluorescence compared to the chelating agents of U.S. Patent 4,637,988. This is especially true of quater-and quinquepyridines covered by the above general formula. Moreover, some of the chelating agents covered by the general formula are excellent intermediates for attaching other desirable groups such as proteins. Others have higher binding constants for Europium (Eu 3).

The present invention also provides a new class of specific binding reagents, such as antigens, enzymes, hormones and the like bearing these highly useful fluorescent labels.

The reagents are formed by adsorbing or covalently binding the fluorescent labeled antigens, haptens, antibodies, plant lectins, carbohydrates, hormones, enzymes and other such species-specific materials.
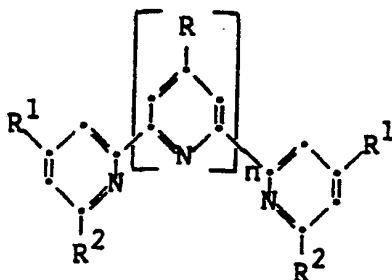
Eu^{+3} is complexed with the chelating agent which is a triplet sensitizer having a triplet energy greater than that of Eu^{+3}. Examples of triplet sensitizers useful herein include heterocyclic and aromatic nitrogen-containing compounds such as quaterpyridines, quinquepyridines, terpyridines and phenanthrolines.

The terpyridines, quaterpyridines and quinquepyridines have the structure

; wherein

R represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, aryloxy, a heterocyclic group, an enzyme, an antigen or an antibody;

R^1 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody;

R^2 represents carboxy, hydroxy carbonyliminodiacetic acid, methyleneimino diacetic acid, hydra zinylylidene diacetic acid or the salts or esters of such acids; and

n is 1 to 4.
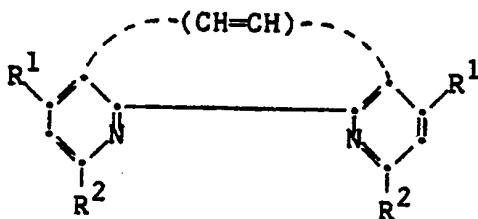
The phenanthrolines have the structure

; wherein

R^1 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody; and

R^2 represents carboxy, hydroxy carbonyliminodiacetic acid, methyleneimino diacetic acid, hydra zinylylidene diacetic acid or the salts of such acids.

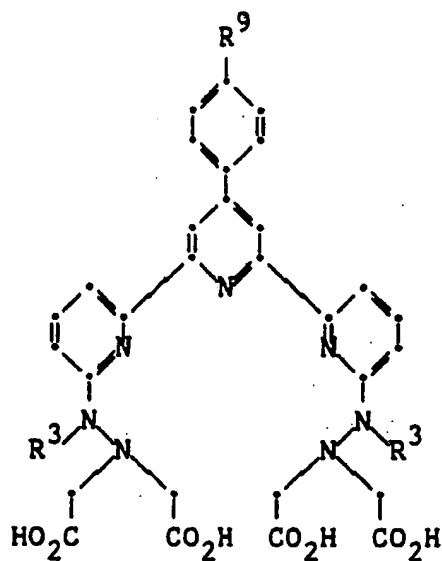With europium, the triplet energy must be at least about 47 Kcal.

The complex contains any ratio of Eu^{+3} metal to chelating agent. In preferred embodiments, the mole ratio of Eu^{+3} to chelating agent is from 1:1 to 2:1. Especially preferred are complexes having a mole ratio of 1:1.

Eu^{+3} and the chelating agent are easily complexed by merely mixing an aqueous solution of the chelating agent with a Eu^{+3} salt in an aqueous solution of pH 7 to 10. The Eu^{+3} salt is any water soluble salt of the metal such as $EuCl_3 \bullet 6H_2O$ or other halogen salts. The chelate is generally prepared in aqueous solution at a pH of between 8 and 11 and preferably 8 and 9. The chelate optionally is mixed with buffers such as phosphate and borate to produce the optimum pH.
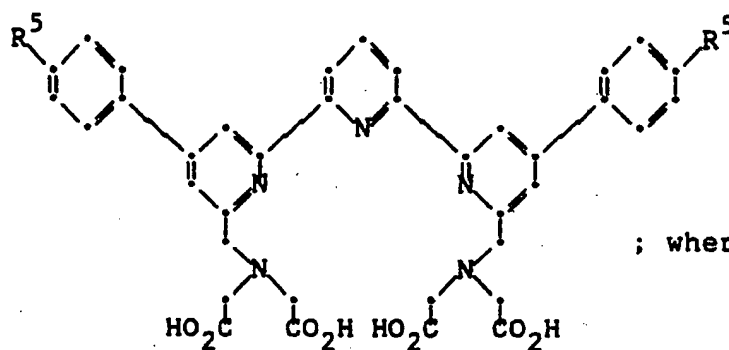
Especially preferred chelating agents are

1)

R^9

; wherein

HO_2C CO_2H CO_2H CO_2H

R^3 represents H, or alkyl of about 1 to 8 carbon atoms, and
R^9 represents H, -OCH$_3$, -NO$_2$; and

2)

R^5 R^5

; wherein

HO_2C CO_2H HO_2C CO_2H

R^5 represents NO$_2$ or-OCH$_3$; and

3)

R^9

; wherein

HO_2C CO_2H HO_2C CO_2H

R9 represents H, $-OCH_3$, $-NO_2$.

The chelates are used to label a variety of physiologically active materials by binding said materials to the chelate by adsorption or by covalent bonding. Among the physiologically active materials which are labeled in this fashion are enzymes and their substrates, antigens, antibodies, i.e. any substance which is capable, under appropriate conditions, of reacting specifically in some detectable manner with an antibody, carbohydrates, metabolites, drugs, other pharmacalogical agents and their receptors and other binding substances. Specific binding assay reagents are described in U.S. Patents 3,557,555; 3,853,987; 4,108,972 and 4,205,058.

Techniques for performing such binding of physiologically active materials to the complexes are those well-known in the art and include simply mixing the materials together.

In specific binding assay methods, a compound having structural similarity to the analyte being determined is herein referred to as the ligand and the labeled compound as the ligand analog. Compounds which specifically recognize the ligands and ligand analogs and bond to them are referred to as receptors.

In performing one such type of assay, the ligand is placed in competition with the ligand analog for binding to the receptor. Unknown concentrations of the ligand are inferred from the measured signal of the labeled, ligand analog. The reaction proceeds as follows:

ligand + (labeled) ligand analog + receptor ⇌ Ligand/receptor + ligand analog/receptor

For illustrative purposes, the discussion which follows describes one particular type of specific binding assay technique, a competitive binding fluorescence immunoassay technique.

This system consists of antigen labeled with a fluorescent label of the present invention, unlabeled native antigen (in test sample) and specific antibody whereby there is competition between the unlabeled antigen and the labeled antigen for binding to the antibody.

The greater the concentration of unlabeled antigen from the test sample in the system, the less the labeled antigen will be bound by the antibody. If the concentrations of labeled antigen and antibody are fixed and the only variable is the level of unlabeled antigen, it is possible to determine the unknown level of unlabeled antigen by physically separating the antigen-antibody complex from the remaining free antigen (both labeled and unlabeled) and comparing the fluorescence of the labeled antigen, either free or bound, with a standard curve plotting of the values given by a range of known amounts of the antigen treated in the same manner.

Once prepared as described hereinabove, the fluorescent-labeled, physiologically active species is useful in fluorescent specific binding assays, particularly those which utilize temporal resolution of the specific detecting signal to distinguish from background as described in German OLS 2,628,158. In this time-resolved mode (i.e. temporal resolution), the sample is excited in an intermittent fashion and information is accepted only during the dark cycle when the long-lived fluorescent label is still emitting strongly but when other sources of fluorescence have decayed. Discontinuous excitation is achieved in a variety of ways, including pulsed laser, mechanical chopping of a continuous excitation beam and moving

the sample in and out of the excitation beam. Moreover, discontinuous excitation has the advantage of allowing the use of high radiant power without the absorption of a large amount of energy by the sample, thus diminishing the probability of sample photodegradation.

Examples of such fluorescent specific binding reagents described herein find utility are described in U.S. Patents 3,988,943; 4,020,151; 3,939,350; 4,220,450 and 3,901,654.

In a preferred embodiment, the specific binding assay is carried out in a dry analytical element such as described in copending U.S. Patent 4,258,001 granted March 24, 1981. In this embodiment, the element contains a support and a spreading reagent layer comprised of polymeric beads, and optionally a registration layer. In some cases, the spreading layer is separate from the reagent layer. The spreading, reagent and registration layers optionally comprise the polymeric bead structure. The polymeric beads of the reagent layer have receptors such as antibodies adsorbed to their surfaces.

The chelate label of the present invention is placed above, below, or in the reagent layer in a manner that prevents the specific reaction from occurring prior to sample wetting, or it is spotted onto the element concurrently with or subsequent to the sample. It is only necessary that the labeled ligand analog permeate the element upon wetting subsequently to compete with the unknown amount of ligand in the sample in the formation of the ligand receptor complex. The assay is performed by fluorimetrically determining the amount of free labeled ligand analog present or the amount of bound labeled ligand analog-receptor complex.

The following non-limiting examples will serve better to illustrate the successful practice of the instant invention.

The syntheses of terpyridines, quaterpyridines and quinquepyridines are similar. The ^{1}H-NMR spectra, the mass spectra, and the infrared spectra were consistent for the desired products in each of the following examples.

SCHEME I
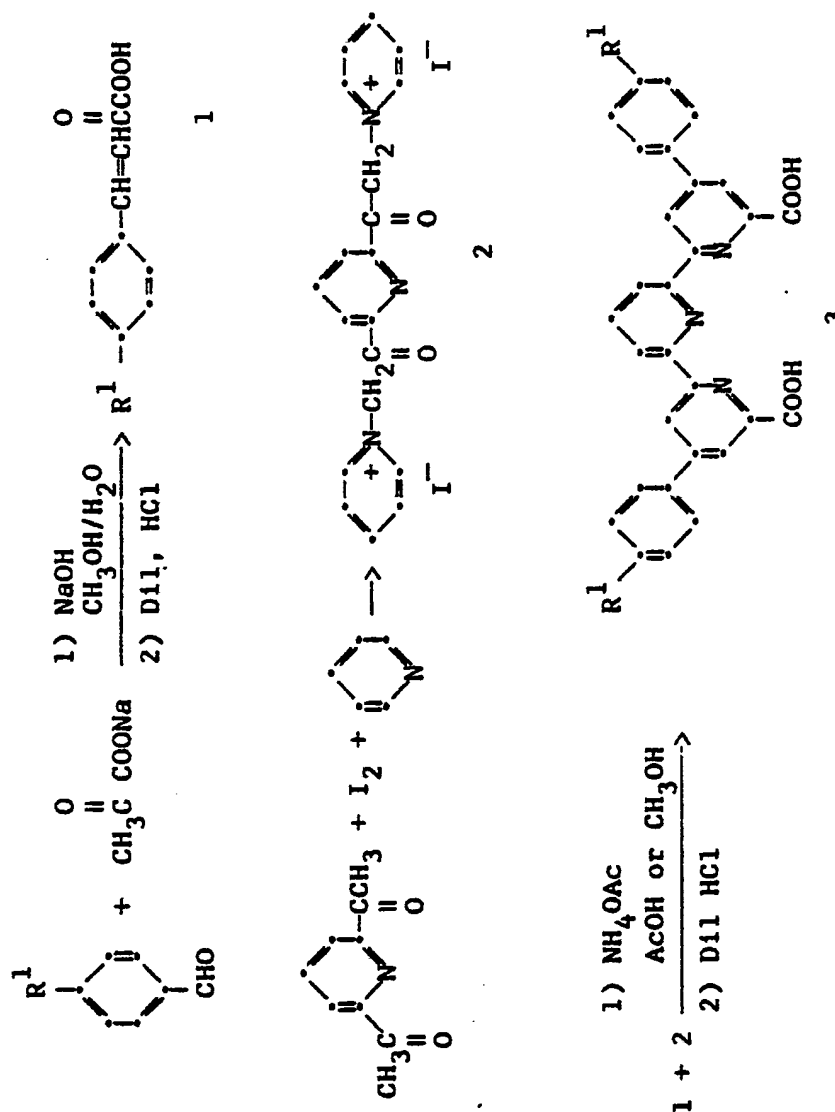
Terpyridine compounds are produced according to the following reaction scheme. AcO represents acetoxy, CH_3COO.
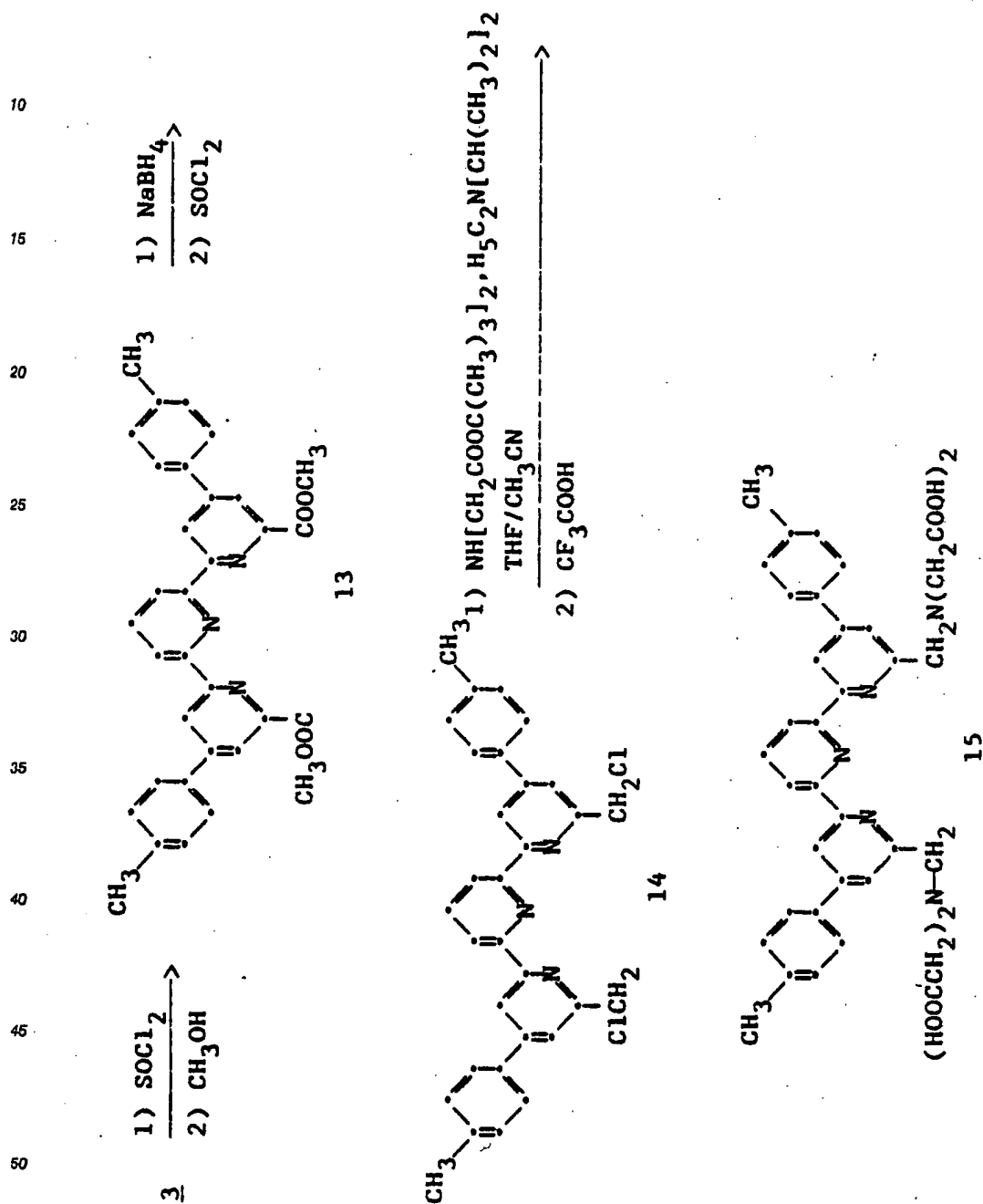
$$R^1\text{—}C_6H_4\text{—}CHO + CH_3\overset{O}{\overset{\|}{C}}COONa \xrightarrow[\text{2) Dil. HCl}]{\begin{array}{c}\text{1) NaOH}\\ CH_3OH/H_2O\end{array}} R^1\text{—}C_6H_4\text{—}CH=CHCCOOH \quad \underset{\mathbf{1}}{\overset{O}{\|}}$$

$$\underset{}{\overset{O}{\underset{\|}{CH_3C}}}\text{—}C_5H_3N\text{—}CCH_3 + I_2 + C_5H_5N \longrightarrow$$

$$\underset{\mathbf{2}}{[\text{N}^+\text{-CH}_2\text{-C-}C_5H_3N\text{-C-CH}_2\text{-N}^+]\ 2\,I^-}$$

$$1 + 2 \xrightarrow[\text{2) Dil HCl}]{\begin{array}{c}\text{1) }NH_4OAc\\ AcOH \text{ or } CH_3OH\end{array}}$$

$$\underset{\mathbf{3}}{R^1\text{—}C_6H_4\text{—}(C_5H_2N\text{-COOH)}_2\text{-}C_6H_4\text{—}R^1}$$

Preparation of 4,4''-Bis(4-methoxyphenyl)-6,6''-dicarboxy-2,2':6,2''-terpyridine

A mixture of compound 1 wherein R^1 is methoxy (7.69 g, 34.9 mmol) and the bispyridinium salt 2 (10.0 g, 17.5 mmol) was refluxed for 18 hours in 600 mL of MeOH with 15 g of NH₄OAc according to the procedure of F. Kr || nke, Synthesis, 1, 1-24 (1976). The solution was filtered and the solid was triturated with hot dilute HCl. The resulting terpyridine diacid was collected by filtration, washed with MeOH then Et₂O (diethyl ether) and dried to give 4.74 g of product as a white powder (51%).
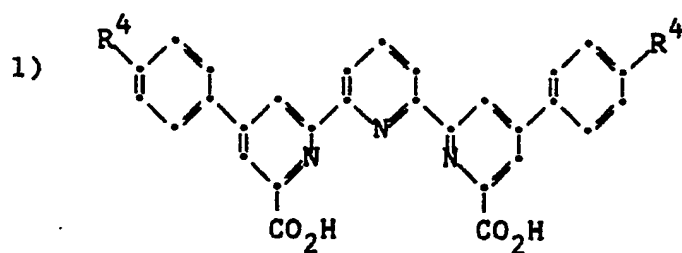
Anal. Calcd. for $C_{31}H_{23}N_3O_6 \cdot H_2O$:
C, 67.5; H, 4.6; N, 7.8 Found: C, 67.8; H, 4.4; N, 7.5.

Terpyridine compounds 13, 14 and 15 were produced from compound 3 in which R^1 was $-CH_3$, according to the following reaction scheme. THF is tetrahydrofuran.
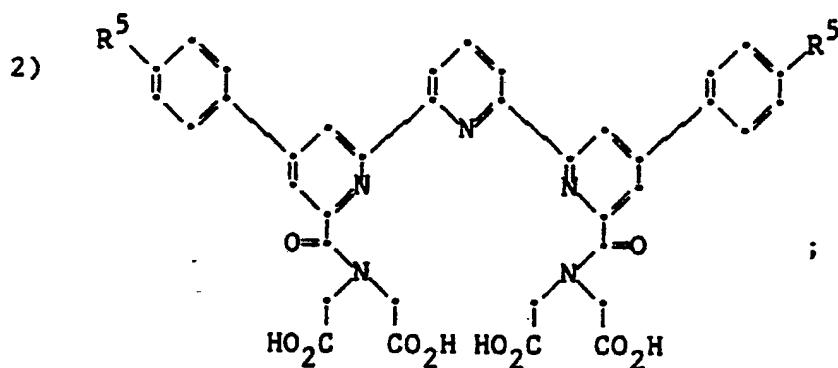

Other representative examples of useful terpyridines are presented in Table 1.
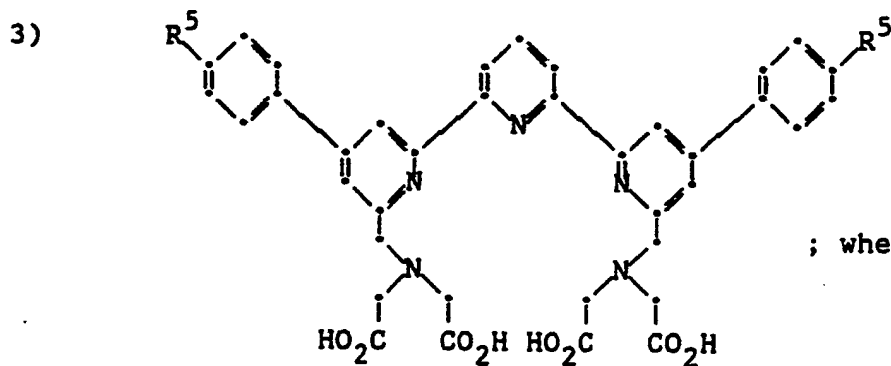
TABLE 1

1)

; wherein

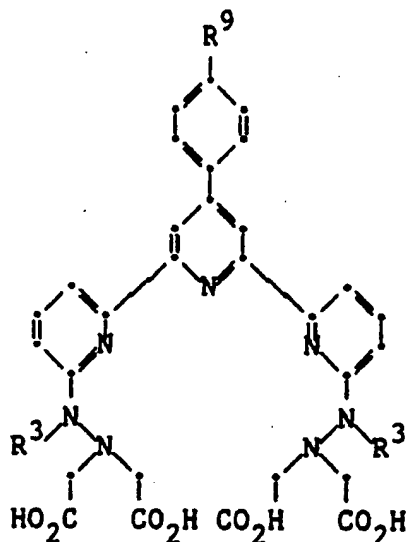R^4 represents $-H$, $-SCH_3$, $-OCH_3$, $-CN$, $-NO_2$, $-CO_2CH_3$, etc.;

2)

; wherein

R^5 represents $-OCH_3$, $-NO_2$, etc.;

3)

; wherein

R^5 represents $-CH_3$, $-OCH_3$, $-NO_2$, etc;

4)
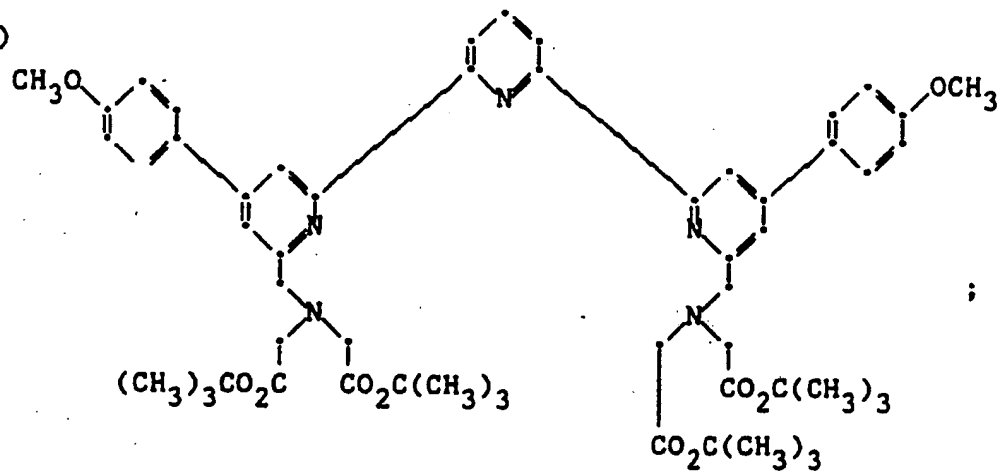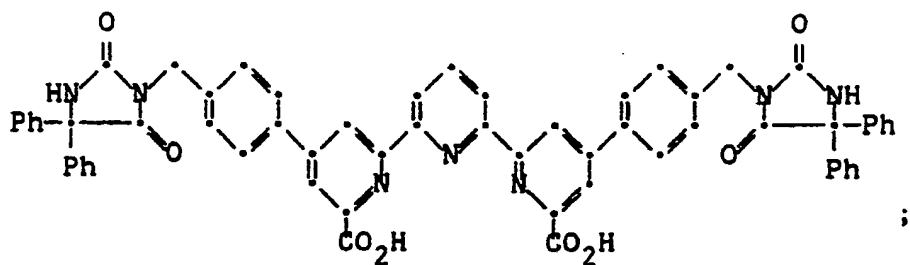

; wherein

R^3 represents H, alkyl of 1 to about 8 carbon atoms; and
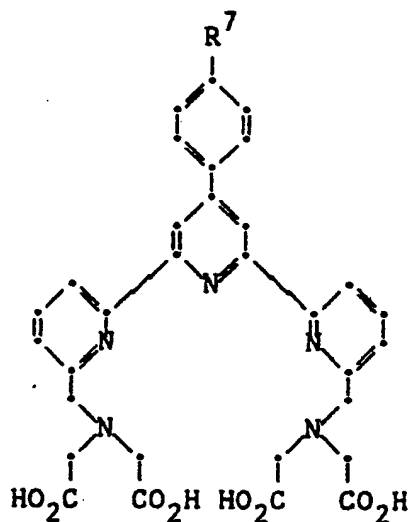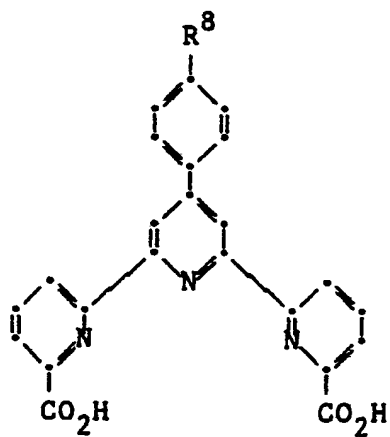
R^9 represents H, $-OCH_3$, $-NO_2$, etc.;

5)

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6)

;

7)

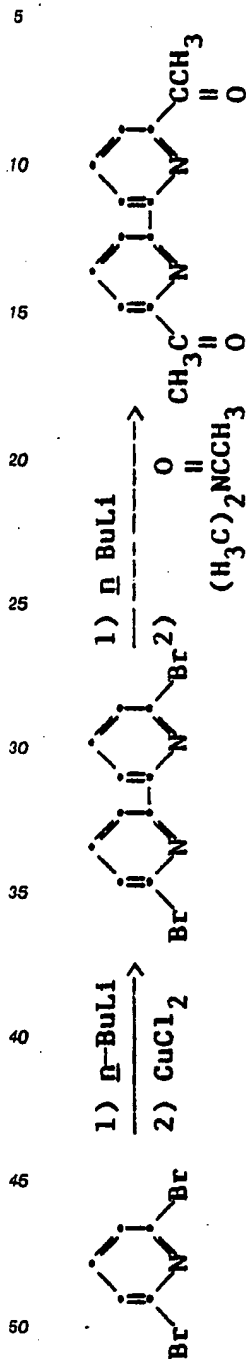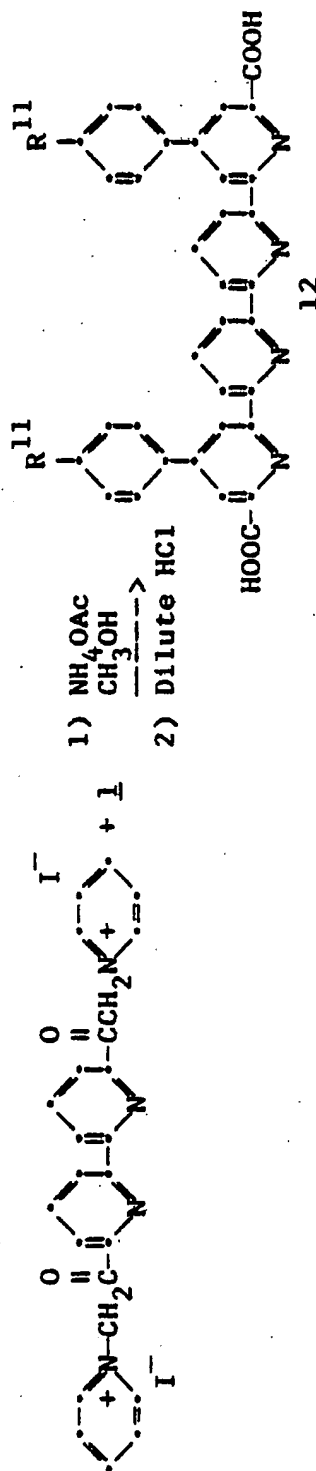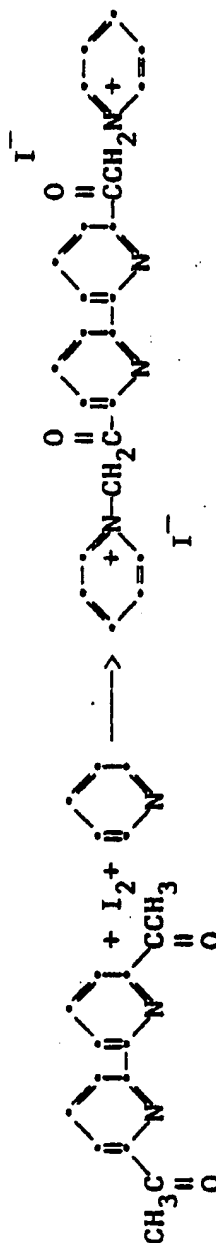; wherein

R^7 represents H, $-OCH_3$, $-NO_2$, etc.;

8)

; wherein

R^8 represents H, $-OCH_3$, $-NO_2$, etc.

Quaterpyridine compounds were produced according to the following scheme. Bu is butyl.

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$\xrightarrow[\text{2) CuCl}_2]{\text{1) n-BuLi}}$

$\xrightarrow[\text{2) (H}_3\text{C)}_2\text{NCCH}_3]{\text{1) n BuLi}}$

$+ I_2 +$

$\xrightarrow[\text{2) Dilute HCl}]{\text{1) NH}_4\text{OAc CH}_3\text{OH}}$

12

12a, R^{11} = —CH$_3$

12b, R^{11} = —OCH$_3$

12c, R^{11} = —NO$_2$

Example 2

13

Preparation of 4.4″-Bis(4 methylphenyl)-6,6″-dicarboxy-2,2′:6′,2″:6″,2‴-quaterpyridine

6,6′-Diacetyl-2,2′-bipyridine was synthesized by the method of J.E.Parks, B.E.Wagner and R.H.Holm, J. Organomet Chem. 56, 53-66 (1973). The bispyridinium iodide was generated by refluxing 2.00 g of the diacetylbipyridine (8.33 mmol) in 50 mL of pyridine with 4.23 g of I_2 (16.7 mmol) for 1 hour. The solution was cooled and filtered, and the solid was washed first with pyridine then with Et_2O (diethyl ether) to give, after drying, 4.46 g of the desired product as a sand-colored solid (82%).

Anal. Calcd. for $C_{24}H_{20}I_2N_4O_2$: C, 44.3; H, 3.1; N, 8.6. Found: C, 44.4; H, 3.2; N, 8.8.

The bispyridinium salt (3.00 g, 4.62 mmol) and 1, with R^{10} being methyl, (1.75 g, 9.23 mmol) were combined and refluxed together with 20 g of NH_4OAc in 500 mL of MeOH for 16 hours. The solution was cooled and filtered. The solid was worked up by the method used for 3 (Example 1), then recrystallized from N,N dimethylformamide (DMF) to give 0.80 g of the titled compound as a cream-colored powder (30%).

Anal. Calcd. for $C_{36}H_{26}N_4O_4 \bullet H_2O$: C, 72.5; H, 4.7; N, 9.4. Found: C, 72.4; H, 4.4; N, 9.3.

Quaterpyridine compounds made by the procedure in Example 2 includes: R^{11} = -CH$_3$ and -OCH$_3$.

Quinquepyridine compounds were prepared according to the following reaction scheme:
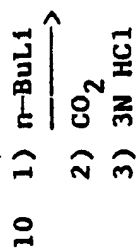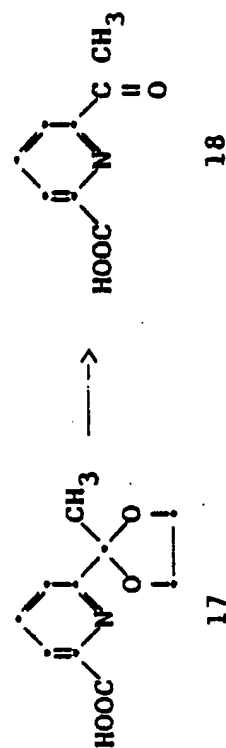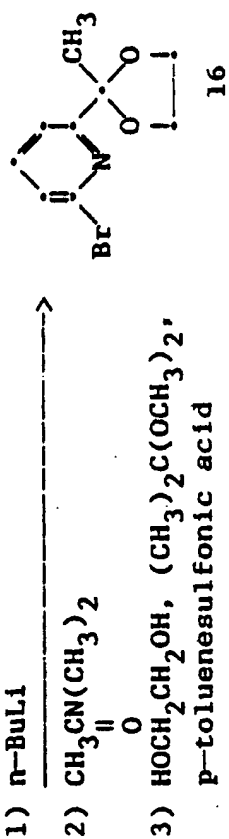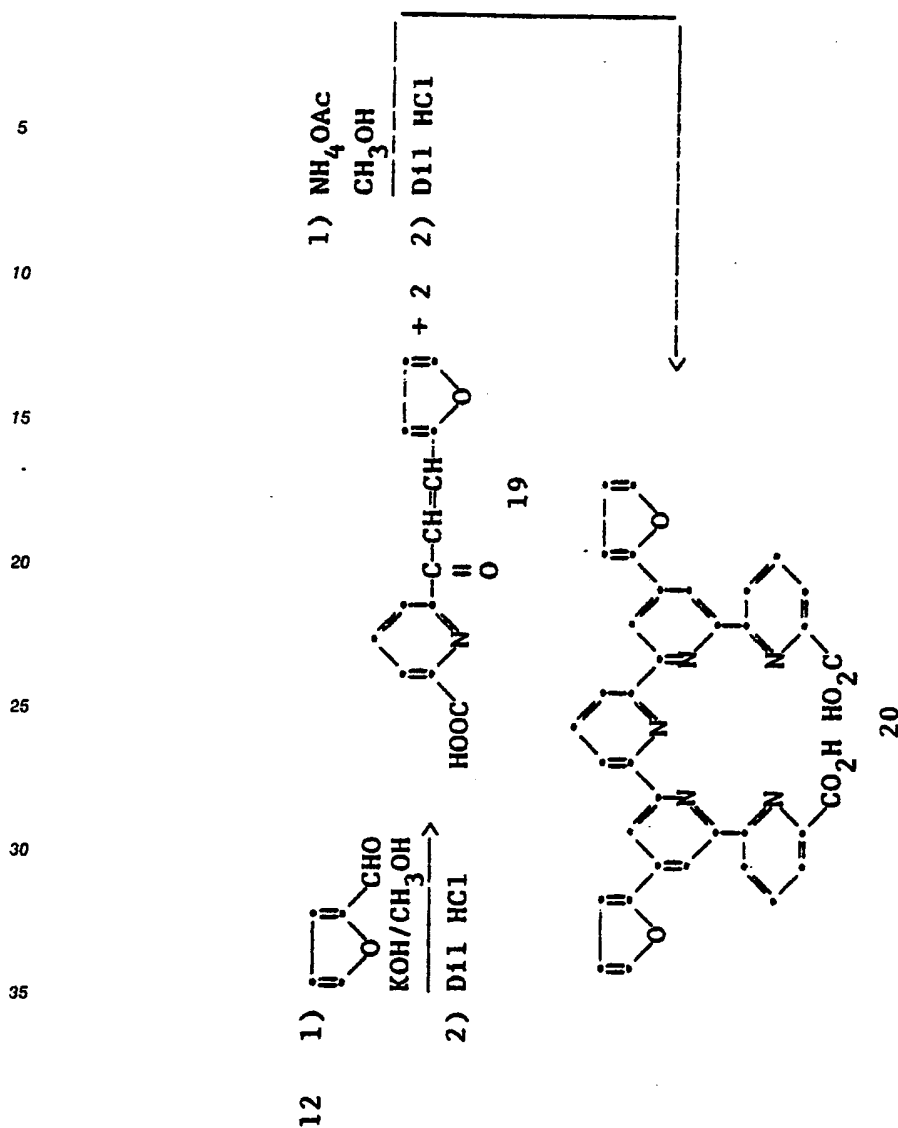
1) n-BuLi

2) $CH_3CN(CH_3)_2$
    $\overset{\|}{O}$

3) $HOCH_2CH_2OH$, $(CH_3)_2C(OCH_3)_2$,
    p—toluenesulfonic acid

16

17

18

10 1) n-BuLi

    2) CO_2

    3) 3N HCl

1) NH$_4$OAc
CH$_3$OH

2) Dil HCl

+ 2

HOOC

—C—CH=CH—
O

19

12

1) CHO
KOH/CH$_3$OH

2) Dil HCl

CO$_2$H HO$_2$C

20

Example 3

Preparation of 4′,4″-Bis(2-Furyl) 6,6″-dicarboxy-2.2′:6′,2″:6′,2″:6″,2″-quinquepyridine (20)

Compound 16, available by the previously cited Parks et al method, (18.1 g, 74.2 mmol) was dissolved in 400 mL of Et$_2$O which had been freshly distilled from LiAlH$_4$. With the temperature maintained at 50°C, 35.3 mL of 2.1 M n-BuLi (74.2 mmol) was added dropwise with stirring under N$_2$. After stirring an additional 0.5 hour at -50°C, dry CO$_2$ (g) was bubbled through the solution and the temperature was allowed to rise to 22°C. After 2 hours, the mixture was extracted with dilute aqueous NaHCO$_3$. The organic phase was washed with water and the combined aqueous phase was acidified to pH 2 with HCl then extracted with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ phase was washed twice with water then dried over Na$_2$SO$_4$. Filtration followed by addition of ligroin, and solvent removal gave a yellow solid. Recrystallization from cyclohexane gave 17 as an off-white solid (7.2 g, 46%).

Anal. Calcd. for $C_{10}H_{11}NO_4$: C, 57.4; H, 5.3; N, 6.7. Found: C, 57.2; H, 5.3; N, 6.7.

Compound 17 (6.1 g, 29.2 mmol) was refluxed In 100 mL of 3N HCl for 2 hours under N_2. The resulting solution was treated with $NaHCO_3$ to pH 2 then extracted 3 times with CH_2Cl_2 The combined organic phase was dried over Na_2SO_4, filtered, and evaporated to give 4.4 g of 18 as an off-white powder (91%).

Anal. Calcd. for $C_8H_7NO_3$: C, 58.2; H, 4.3; N, 8.5. Found: C, 58.0; H, 4.3; N, 8.4.

Compound 18 (0.50 g, 3.0 mmol) was refluxed with 2-furaldehyde (0.29 g, 3.0 mmol) in 25 mL of MeOH with 0.2 g of KOH under N_2 for 1.5 hours. The solution was poured into 100 mL of aqueous $NaHCO_3$ and extracted with CH_2Cl_2 which was discarded. The aqueous phase was acidified and extracted twice with CH_2Cl_2. The organic phase was worked up by the procedure used for compound 18 to give chalcone 19 as a light green foam which was pure enough to continue with.

Chalcone 19 (0.60 g, 2.5 mmol) and 2 (0.71 g, 1.2 mmol) were combined and refluxed together with 5 g of NH_4OAc in 100 mL of MeOH for 16 hours. The solution was cooled and filtered and the resulting solid was worked up by the procedure used for the terpyridine diacid 3 in which R^{10} is $-OCH_3$, to give 20 as a tan powder (0.40 g 53%).

Anal. Calcd. for $C_{35}H_{21}N_5O_6 \cdot 1/2 H_2O$: C, 68.2; H, 3.6; N, 11.4. Found: C, 68.3; H, 3.5; N, 11.5.

A 10^{-6} M solution of the quinquepyridine 20 and $EuCl_3 \cdot 6H_2O$ was highly luminescent under long wavelength UV light.

Example 4

Preparation of fluorescent chelates with ter-, and quater-pyridines and Eu^{+3}

Stock 10^{-5} m solutions of terpyridine. Eu^{+3} complexes were prepared by weighing the appropriate amount of the terpyridine into a 1 l volumetric flask; adding 1.00 mL of a stock 10^{-2}M $EuCl_3$ solution and then diluting with pH 10 borate buffer. For luminescence vs. concentration studies, the dilutions were carried out with 10^{-5} M Eu^{+3} solutions in pH 10 borate buffer. The constant Eu^{+3} concentration forces the terpyridine to remain complexed at low concentrations (Ka = about $5 \times 10^6 M^{-1}$).

Solutions of the quaterpyridine diacid Eu^{+3} complexes were prepared so that the Eu^{+3} concentration was maintained at 10X the concentration of the quaterpyridine diacid.

For solutions of the terpyridine tetraacid $\cdot Eu^{+3}$ chelates, the Eu^{+3} concentration was maintained at twice the ligand concentration.

The luminescence properties of the chelates are Presented in Tables 2, 3 and 4.

TABLE 2

Luminescence Properties of the Ter- and
Quaterpyridine Eu^{+3} Chelates^a

Compound	ε(M^{-1}cm^{-1})*	Φ***	Irel**	
Table I, compound 1; R^4= -CH$_3$	13,000(340nm)	0.08	9.11	(Prior art)
Table I, compound 1; R^4= -OCH$_3$	16,000(340nm)	0.07	12.5	
Example 2, compound 12a; R^{11}= -CH$_3$	8,500(350nm)	0.16	21.5	
Example 2, compound 12b; R^{11}= -OCH$_3$	16,000(350nm)	0.14	35.7	
Example 1, compound 15****	14,000(339nm)	------	18.5	

a. Diacid = 10^{-6}M, [Eu^{+3}] = 10^{-5}M.

* Refers to the extinction coefficients.

** Refers to relative luminescence intensities.

*** Refers to the relative emission quantum efficiencies.

**** Contains some diacid impurity.

TABLE 3

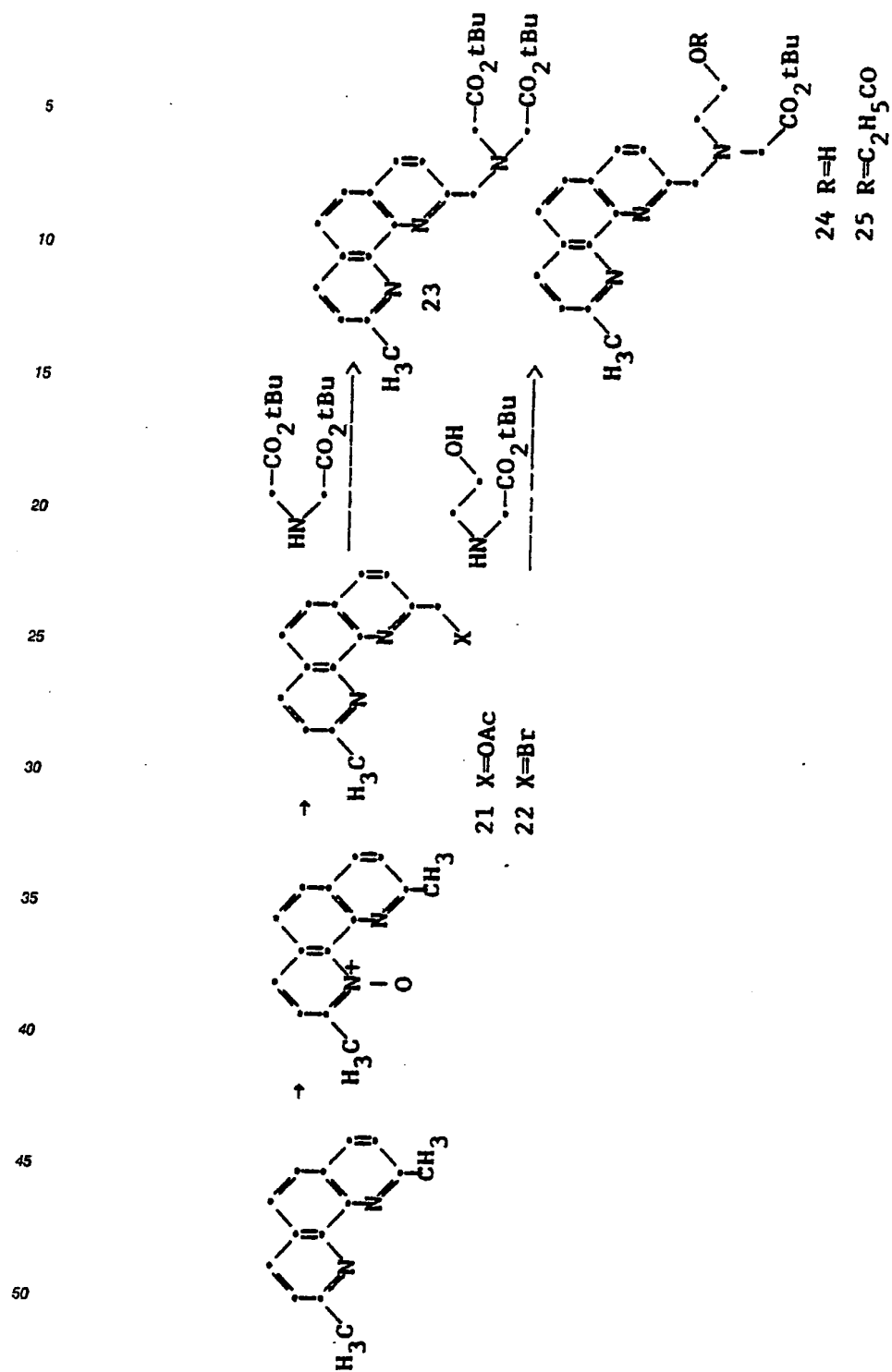### Luminescence vs. Concentration for the Eu^{+3} − Chelate with Compound 1 of Table I (R^4= −CH$_3$)

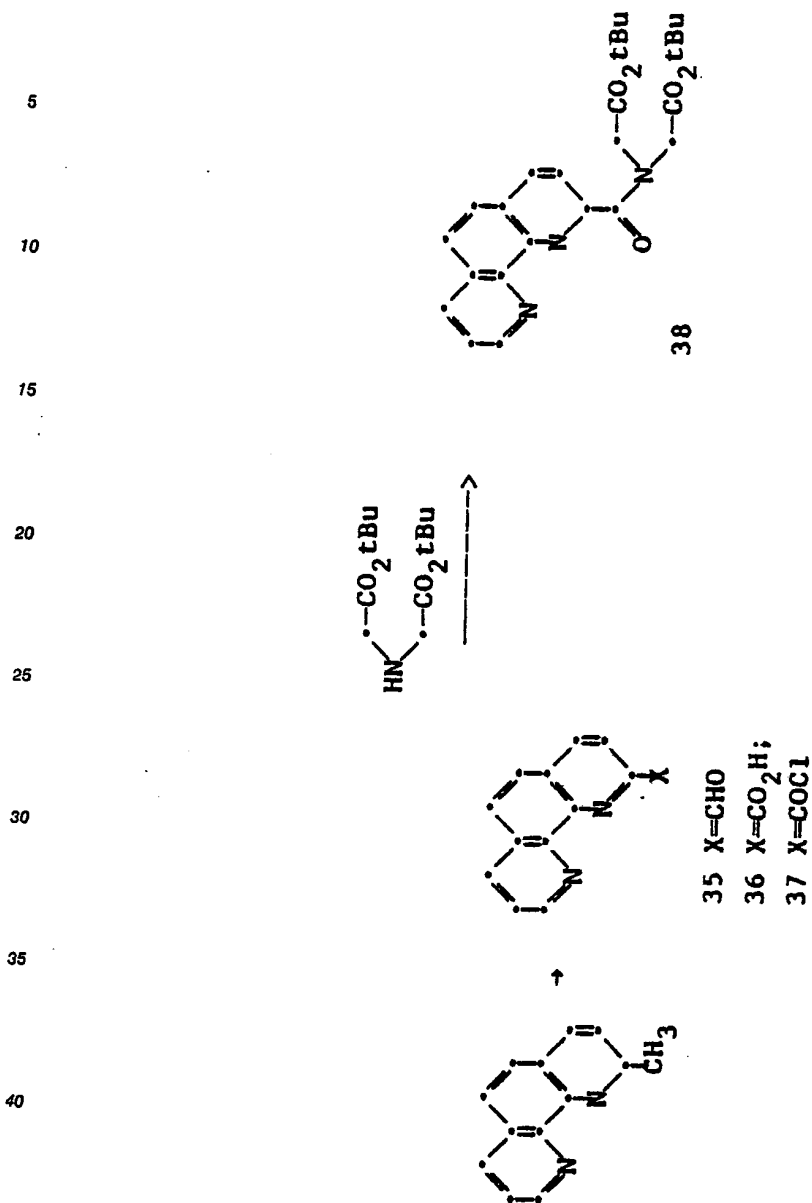| Concentration of Chelate (M)	pH	Luminesence at 614 nm
10^{-6}	10	11.46
5×10^{-7}	10	7.41
10^{-7}	10	0.86
5×10^{-8}	10	0.35
3×10^{-8}	10	0.23
10^{-8}	10	0.07
5×10^{-9}	10	0.03

TABLE 4

Luminescence vs. Concentration for the Quaterpyridine·Eu^{+3} Chelate (Example 2, Compound 12b; R^{11} = −OCH$_3$)

Concentration of Chelate	pH	Luminesence at 616 nm
10^{-5}	10	365
10^{-6}	10	35.66
4×10^{-7}	10	14.26
2×10^{-7}	10	6.46
10^{-7}	10	2.61
4×10^{-8}	10	0.98
2×10^{-8}	10	0.42
10^{-8}	10	0.24
4×10^{-9}	10	0.09

A series of phenanthroline chelating agents were prepared using the following series of reaction - schemes. In the chemical structures tBu refers to tertiary butyl.

CO₂tBu — rendered as: CO_2tBu labels throughout the scheme.

32 Z=H
33 Z=O

34

31

26 X=CHO
27 X=CH₂OH
28 X=CH₂Br
29 X=CO₂H
30 X=COCl

CH₃

H₃C

38

Compound with —CO2tBu, —CO2tBu, N, O substituents on phenanthroline

HN with —CO2tBu, —CO2tBu

35 X=CHO
36 X=CO2H;
37 X=COCl

CH3-substituted phenanthroline

Example 5

Preparation of 2-Bromomethyl-9-methyl-1,10-phenanthroline (22)

A mixture of 6.3 g (0.03 mol) of 2,9-di-methyl-1,10-phenanthroline, 6 mL of 30% H_2O_2 and 30 mL of trifluoroacetic acid (TFA) was refluxed for 3 hours. The reaction was followed by ^{1}H NMR analysis of the reaction mixture. The volatiles were removed using a rotary evaporator initially and then a vacuum pump to yield a mono N-oxide isolated as a golden syrup that crystallized from Et2O as the TFA salt. ^{1}H NMR (CF_3CO_2H) 2.9 (s, 3H); 3.1 (s, 3H); 7.95 (ABq, 1H); 8.1 (ABq, 1H); 8.2 (s, 2H); 8.35 (ABq,1H); 8.9 (ABq,1H).

The N-oxide was dissolved in 90 mL of Ac_2O and then added over 20 min to 250 mL of refluxing Ac_2O. Ac_2O is acetic anhydride. After refluxing for an additional 30 minutes, the dark solution was cooled and the Ac_2O removed in vacuo. The crude residue was purified by chromatography on silica gel using CH_2Cl_2 to elute 2-acetoxymethyl-9-methyl-1,10-phenanthroline (21). ^{1}H NMR ($CDCl_3$) 2.06 (s, 3H); 3.82 (s, 3H); 5.53 (s, 2H); 7.4 (ABq, 1H J = 9 Hz); 7.57 (ABq, 1H, J = 9 Hz); 7.62 (s, 2H); 8.0 (Abq, 1H, J = 9 Hz); 8.13 (ABq, 1H, J 9 Hz).

The acetate (21) was dissolved in 60 mL of 31% HBr in HoAc and refluxed for 4 hours. After cooling, all the solvent was removed in vacuo. The resulting black residue was suspended in a mixture of CH_2Cl_2 and dilute aqueous $NaHCO_3$. Four 200 mL CH_2Cl_2 extracts removed the desired bromide from the insoluble material floating at the solvent interface. After drying over Na_2SO_4 and removal of the solvent, the dark residual solid was relatively pure phenanthroline (22) containing a trace amount of 2,9-dimethyl-1,10 phenanthroline. The dark impurities were removed by chromatography on silica gel using CH_2Cl_2 as the eluent which gave 3.5 g (42%) of 22. ^{1}H NMR ($CDCl_3$) 2.9 (s, 3H); 4.94 (s, 2H); 7.43 (ABq, 1H, J = 9 Hz), 7.6 (s, 2H); 7.8 (ABq, 1H, J = 9 Hz); 7.97 (ABq, J = 9 Hz); 8.1 (ABq, 1H, J = 9 Hz).

Example 6

Preparation of 2-[di-(t-butoxycarbonylmethyl)-aminomethyl]-9-methyl-1,10-phenanthroline (23)

Part A - di-tert-butyl iminodiacetate

A mixture of 5.0 g (38 mmol) of iminodiacetic acid, 4.5 mL (50 mmol) of $HClO_4$ and 100 mL of tert-butyl acetate was stirred for about 20 minutes at 20°C until the reaction became homogeneous. After standing for 3 days, the solution was poured into 10% aqueous $NaHCO_3$ and extracted three times with 50 mL portions of CH_2Cl_2. After drying over Na_2SO_4 and removal of solvents using an aspirator, the resulting oil was a mixture of di-tert-butyl iminodiacetate and tert-butyl acetate. The crude product was dissolved in 50 mL of CH_2Cl_2 and extracted at 0°C with 100 mL of 6 M HCl; the aqueous phase was extracted twice with CH_2Cl_2 and then poured into ice water saturated with $NaHCO_3$. The resulting alkaline solution was extracted three times with CH_2Cl_2. After drying over Na_2SO_4, the volatiles were removed to yield 26, isolated as a yellow oil (2.5 g, 27%) which upon standing at -5°C solidified, ^{1}H-NMR ($CDCl_3$ 1.4 (s, 18H); 2.0 (s, 1H); 3.23 (s,4H). Mass spec m/e 245 (M^+), 133.

Part B - tert-Butyl Ester (23)

A mixture of 900 mg (3.6 mmol) of di-tert-butyl iminodiacetate, 800 mg (2.8 mmol) of bromide (22) and 0.5 g of diisopropylethylamine were stirred in 8 mL of dry DMF at 25°C for 45 hours. The solution was poured into dilute $NaHCO_3$ in deionized H_2O and extracted 3 times with CH_2Cl_2. After drying over Na_2SO_4 and solvent removal, by ^{1}H NMR the residue was a 1:1 mixture of 23 and di-tert-butyl iminodiacetate. The material was chromatographed on florisil; a mixture of 40 percent CH_2Cl_2 in cyclohexane was used to elute the di-tert-butyl iminodiacetate followed by a mixture of the diacetate and 23; CH_2Cl_2 eluted 200 mg of Pure 23. ^{1}H NMR ($CDCl_3$) 1.3 (s, 9H); 2.7 (s, 3H);3.35 (s, 4H); 4.3 (s, 2H); 7.4 (ABq, 1H); 7.5 (s, 2H); 7.6 (ABq, 2H); 8.05 (ABq, 1H); 8.05 (m, 2H). Mass spec (field desorption) m/e 451 (M^+).

Example 7

Preparation of 2-[N,N-di(carboxymethyl)amino-methyl]-9-methyl-1,10-phenanthroline (42 Table II)

A mixture of 200 mg of 23 and 5 mL of trifluoroacetic acid was allowed to stand at 20°C for 1 hour. In order to monitor the reaction by NMR the solvent was removed under vacuum. A ¹H NMR spectrum of the residue redissolved in TFA revealed the t-butyl esters had been removed.

Example 8

Preparation of 2-(N-tert-butoxycarbonylmethyl-N-hydroxyethylaminomethyl)-9-methyl-1,10-phenanthroline (24)

Part A - tert-Butyl N-(2-Hydroxyethyl)glycinate (10)

A mixture of 6 g (0.03 mmol) of tert-butyl bromoacetate in 15 mL of CH_3CN was added over 30 minutes to a 20°C stirred solution of 30 g of 2-aminoethanol in 30 mL of CH_3CN. After stirring for 3 hours, the solvent was removed using a rotary evaporator.

The residue was poured into H_2O and extracted six time with CH_2Cl_2. After drying over Na_2SO_4 and removal of the CH_2Cl_2, 3.1 g (60%) of 10 was obtained as a water-white liquid. ¹H NMR 1.35 (s, 9H); 2.1 (broad s, 1H); 2.67 (t, 2H); 3.18 (s, 2H); 3.5 (t, 2H).

Part B

A solution of 3.1 g (18 mmol) of the glycinate from Part A, 4.0 g (11 mmol) of bromide 22 and 2.5 g (19 mmol) of diisopropylethylamine in 15 mL of CH_2Cl_2 was stirred for 24 hours at 30°C. The solution was poured into dilute $NaHCO_3$ in deionized H_2O and extracted 3 times with CH_2Cl_2. After drying over Na_2SO_4 and removal of the solvent, 3 g of residue was obtained which was essentially clean 24. Chromatography on florisil required 1-10% $MeOH/CH_2Cl_2$ to elute pure 24. ¹H NMR ($CDCl_3$) 1.44 (s, 9H); 2.83 (s, 3H); 2.94 (t, 2H); 3.4 (s, 2H); 3.6 (t, 2H); 7.4 (ABq, 1H); 7.6 (s, 2H) 7.7 (ABq, 1H); 8.0 (ABq, 1H); 8.1 (ABq, 1H).

Example 9

Preparation of 2-(N-tert-butoxycarbonylmethyl-N-propionyloxyethyl)aminomethyl-9-methyl-1,10-phenanthroline (25)

To a mixture of 100 mg of 24 and 0.3 g of triethylamine in 2 mL of CH_2Cl_2 was added 0.2 g of propionyl chloride. The reaction mixture was stirred for 15 hours, poured into dilute $NaHCO_3$ in deionized H_2O and extracted 3 times with CH_2Cl_2. After drying over Na_2SO_4 and removal of the solvent, the residue was chromatographed on florisil using CH_2Cl_2 to elute 110 mg of 25. ¹H NMR ($CDCl_3$) 1.03 (t, 3H); 1.43 (s, 9H); 2.3 (q, 2H); 2.87 (s, 3H); 3.0 (t, 2H); 3.35 (s, 2H); 4.16 (t, 2H); 4.33 (t, 2H); 7.43 (ABq, 1H, J = 9 Hz); 7.65 (s, 2H); 7.97 (ABq, 1H); 8.03 (ABq, 1H); 8.13 (ABq. 1h). Mass spec (FD) m/e 437.

Example 10

Preparation of 2,9-Bisformyl-9,10-phenanthroline (26)

As described by C.J.Chandler, L.W.Deady, and J.A.Reiss, J. Heterocyclic Chem., 18, 599 (1981), a
mixture of 9.0 g (43.2 mmol) of 2,9-dimethylphenanthroline and 22.5 g (0.2 mol) of selenium dioxide were
refluxed in 600 mL of dioxane containing 4% H_2O for 2.5 hours. The hot dark solution was filtered through
Celite®, cooled, and the resulting solid was collected by filtration. After air drying, 8.0 g (80%) of 26
isolated as a yellow solid was obtained. ^1H NMR (DMS)-d_6) 8.2 (s, 2H); 8.32 (ABq, 2H); 8.7 (ABq, 2H) 10.35
(s, 2H).

Example 11

Preparation of 2,9-Bis(hydroxymethyl)-1,10-phenanthroline (27)

To a mixture of 2.0 g of dialdehyde 26 in 40 mL of DMF and 5 Ml of ethanol was added 800 mg of
$NaBH_4$. The solution was stirred 6 hours at 30°C then 5 mL of acetone was added. The solvent was
removed under vacuum and the residue recrystallized from 50 mL of H_2O to yield 1.7 g of diol (27). ^1H
NMR (DMSO-d_6) 4.7 (s, 4H); 5.45 (broad s, 2H); 7.6 (ABq, 2H); 7.7 (s, 2H); 8.25 (ABq, 2H).

Example 12

Preparation of 2,9-Bis(bromomethyl)-1,10-phenanthroline (28)

A mixture of 250 mg of diol 27 in 10 mL of 31% HBr in HOAc was refluxed for 3 hours. The solvent of
the resulting orange solution was removed in vacuo and the residue was taken up in CH_2Cl_2 and dilute
aqueous $NaHCO_3$. Due to low solubility, 3 CH_2Cl_2 extractions were necessary to leach all the dibromide 28
from the insoluble material. After drying over Na_2SO_4 and removal of the solvent, 220 mg (~60%) of 28 was
obtained as a dark solid which was pure by NMR. Chromatography on silica gel using CH_2Cl_2 eluted 28 as
a white solid. ^1H NMR ($CDCl_3$) 4.87 (s, 4H); 7.63 (s, 2H); 7.83 (ABq, 2H, J = 8 Hz); 8.13 (ABq, 2H, J = 8
Hz).

Example 13

Preparation of 2,9-Bis[N,N-ditert-butoxycarbonyl-methylaminomethyl]-1,10-phenanthroline (32)

A mixture of 220 mg (0.57 mmol) of dibromide 28, 400 mg (1.6 mmol) of di-tert-butyl iminodiacetate,
0.5 g of diisopropylethylamine in 5 mL of 1:1 CH_2Cl_2/CH_3CN was stirred for 15 hours at 25°C. The solution
was poured into deionized H_2O/Na_2HCO_3 and extracted three times with CH_2Cl_2. After drying over Na_2SO_4
and solvent removal, the residue was chromatographed on florisil. Residual iminodiacetate and tetraester
were eluted with 20-50% CH_2Cl_2 in cyclohexane; 5% $MeOH/CH_2Cl_2$ eluted 120 mg of clean 32. ^1H NMR
($CDCl_3$) 1.30 (s, 18H); 1.36 (s, 18H); 3.42 (s, 8H); 4.23 (s, 2H); 4.33 (s, 2H); 7.60 (s, 2H); 8.05 (ABq, 4H).

Example 14

<u>Preparation</u> <u>of</u> <u>1,10-Phenanthroline-2,9-dicarboxylic</u> <u>acid</u> <u>Chloride</u> <u>(30)</u>

5   Following the procedure of Chandler et al, referred to in Example 10, a solution of 0.6 g (2.5 mmol) of bisaldehyde 26 in 12 mL of 80% nitric acid was refluxed for 3 hours. Upon cooling, the reaction was poured onto ice and the diacid 29 was collected by filtration. Upon air drying, 0.5 g (75%) of 18 was isolated ^{1}H-NMR (DMSO-d_6) 8.20 (s, 2H); 8.45 (ABq, 2H); 8.75 (ABq, 2H).

     A mixture of 0.5 g of dicarboxylic acid 29 was refluxed in 5 mL of thionyl chloride for 5 hours. The suspension slowly went into solution as the reaction proceeded. The volatiles were removed using a rotary
10 evaporator; the residue was twice dissolved in benzene and stripped to dryness.

     ^{1}H NMR (CDCl$_3$) 8.05 (s, 2H); 8.45 (s, 4H).

15   <u>Example 15</u>

    <u>Preparation</u>   <u>of</u>   <u>N,N′-Bis(tert-butoxycarbonylmethyl)-N,N′-Bis(2-hydroxyethyl)-1,10-phenanthroline-2,9-dia-mide (34)</u>
20

     To a 20°C mixture of 400 mg (4 mmol) of Et$_3$N and 0.5 g (3 mmol) of tert-butyl N-(2-hydroxyethyl)-glycinate in 3 mL of CH$_2$Cl$_2$ added 0.3 g (1 mmol) of the bisacid chloride 30. The solution was stirred overnight, poured into Na$_2$HCO$_3$/H$_2$O and extracted three times with CH$_2$Cl$_2$. After drying over Na$_2$SO$_4$ and
25 removal of solvent, the residue was chromatographed on florisil. The product came off mainly with CH$_2$Cl$_2$-5% MeOH/CH$_2$Cl$_2$ but was contaminated with residual glycinate. This material was slurried in 4 mL of Et$_2$O, 1 mL of petroleum ether was added and the solution decanted from the clean product 20. ^{1}H NMR (CDCl$_3$) 1.13 (s, 9H); 1.4 (s, 9H); 3.79 (m, 8H); 4.2 (s, 2H); 4.3 (s, 2H); 7.83 (s, 2H); 7.87 (ABq, 1H); 8.35 (m, 3H). Mass spec (FD) m/e 582.
30

    <u>Example 16</u>

35   <u>Preparation of N,N,N′,N′-tetra(tert-butoxycarbonylmethyl)-1,10-phenanthroline-2,9-diamide (33)</u>

     A mixture of 3.0 g (11.2 mmol) of diacid 29 and 100 mL of thionyl chloride was refluxed for 16 hours, cooled, and stripped under vacuum. The crude bis(acid chloride) 30, without further purification, was
40 suspended in 200 mL of dry THF to which was added 6.03 g (24.6 mmol) of di-t-butyl iminodiacetate, followed by 3.4 mL (24.6 mmol) of triethylamine. After stirring at 200°C for 2 hours, the solution was filtered. The filtrate, after concentration, was chromatographed on florisil using a gradient from 25% EtOAc/CH$_2$Cl$_2$-EtOAc to elute 3 g (37%) of 33 as a clean oil which formed a stiff foam. ^{1}H NMR (CDCl$_3$) 1.13 (s, 18H); 1.53 (s, 18H); 4.37 (s, 4H); 5.07 (s, 4H); 7.8 (s, 2H); 8.17 (ABq, 2H, J = 8 Hz); 8.27 (ABq, 2H,
45 J = 8 Hz). Mass spec (FD) m/e 723 (m$^+$).

    Anal. Calcd. for C$_{38}$H$_{50}$N$_4$O$_{10}$: C, 63.1; H, 7.0; N, 7.8. Found: C, 62.8; H, 7.0; N, 7.9.

    <u>Example 17</u>
50

55

Preparation of N,N,N',N'-tetra(carboxymethyl)-1,10-phenanthroline-2,9-diamide (39, Table 5)

A mixture of 800 mg (1.1 mmol) of 21 in 25 mL of TFA stood for 1 hour at 20°C. Upon removal of the
solvent under vacuum and washing of the residue with Et_2O, 0.54 g of product (98%) was obtained, isolated
as a white power. ^{1}H NMR ($CDCl_3$/DMSO-d_6) 4.32 (s, 4H); 4.84 (s, 4H); 8.05 (ABq, 2H, J = 8 Hz); 8.1 (s,
2H); 8.57 (ABq, 2H, J = 8 Hz). Anal Calcd. for $C_{22}H_{18}N_4O_{10}$ 1/2 H_2O: C, 52.1; H, 3.8; N, 11.0. Found: C,
52.1; H, 3.9; N, 11.0.

Example 18

Preparation of N,N-Bis(tert-butoxycarbonylmethyl)-1,10-phenanthroline-2-carboxylic acid amide (38)

A mixture of 200 mg (0.9 mmol) of 9,10-phenanthroline-2-carboxylic acid in 10 mL of thionyl chloride
was refluxed for 6 hours. Although the reaction never became homogeneous, the volatiles were removed
using a rotary evaporator. The residue was suspended in benzene and stripped to dryness. The crude
product, dissolved in 10 mL of CH_2Cl_2, was combined with 240 mg (1 mmol) of di-tert-butyl iminodiacetate
followed by 300 mg of triethylamine. After stirring at 30°C for 24 hours, the reaction was poured into
NaHCO3/deionized H_2O and extracted three times with CH_2Cl_2, dried over Na_2SO_4 and evaporated. The
residue was chromatographed on florisil using 1-5% MeOH/CH_2Cl_2 to elute 100 mg (24%) of the desired
amide 38. ^{1}H NMR ($CDCl_3$) 1.1 (s, 9H); 1.37 (s, 9H); 4.2 (s, 2H); 4.63 (s, 2H); 7.5 (d, 1H); 7.65 (s, 2H); 8.0
(d, 1H); 8.03 (d, 1H); 8.13 (d, 1H); 9.0 (m, 1H). Mass spec (FD) m/e 451.

Hydrolysis Proceeded quantitatively by the procedure used for compound 39, Table 5, to give diacid
compound 43, Table 5. Other similarly produced compounds are also presented in Table 5.

TABLE 5

Chelating Agents

OH
CO₂H

41

OCOEt
CO₂H

40

CO₂H
CO₂H

43

CO₂H
CO₂H

39 (Prior art)

CO₂H
CO₂H

42

Example 19

Preparation of phenanthroline tetraacid complexes of Eu^{+3}

Stock 10^{-4}M solutions of the phenanthroline chelating agents were prepared by weighing the appropriate amounts into 1L volumetric flasks, adding 10 mL of 10^{-2}m EuCl$_3$ solution and diluting with pH 10 borate buffer. In the case of compound 43, Table 5, the solution reached constant luminescence immediately. With compound 39, Table 5, it was necessary to allow the solution to stand for eight days to reach constant luminescence. Relative quantum efficiencies (Φ), absorption maxima and luminescence of the chelates prepared in this example are presented in Tables 6 and 7.

TABLE 6

Quantum Efficiencies and Absorption Maxima for Some Eu^{+3} Chelates in pH 10 Borate Buffer

	ΦEu^{+3} Chelate	λmax
	0.02	291

39, Table 5 (Prior art)

	0.04	277

42, Table 5

	0.06	277

43, Table 5

TABLE 7
Luminescence vs. Concentration for the Eu^{+3}
Chelates of Phenanthroline Tetraacids 39 and 43

Table II

Chelating Agent	$-Log[L]^{\underline{a}}$	$Log\ I^{\underline{b}}$
39	4.1	-0.48
39	5.2	-1.50
43	4.2	1.36
43	5.2	0.42
43	6.2	-0.60
43	7.2	-1.52

a. $[Eu^{+3}] = [L]$

b. λexcitation 330nm, λemission 614nm in
   pH 8.5 borate buffer

Claims

1. A fluorescent chelate of Eu^{+3} and a chelating agent having the general structure
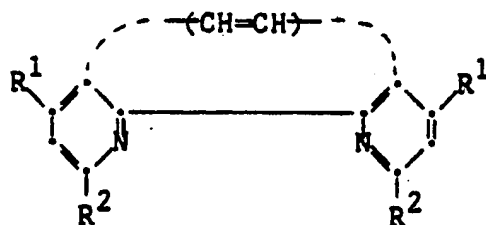

; wherein

R represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, aryloxy, a heterocyclic group, an enzyme, antigen, or antibody;

R^1 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody;
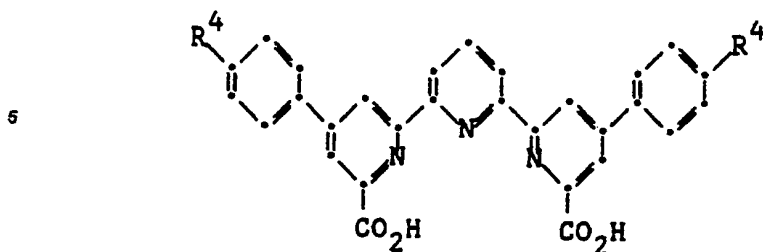
R^2 represents carboxy, hydroxy, carbonyliminodiacetic acid, methyleneiminodiacetic acid, hydra zinylylidenediacetic acid or the esters or salts of such acids;

n is 0 to 4; and

m is 0 to 1 provided that a) m can be 1 only when n is 0 and b) compounds of the structures (i), (ii) or (iii) are excluded from said general structure.

(1)

(ii)

(iii)

2. A fluorescently labeled specific binding reagent comprising a physiologically active material adsorbed or bound to a fluorescent label comprising a fluorescent chelate of a Eu^{+3} and a chelating agent as claimed in Claim 1.

3. The invention of claim 1 or 2 wherein the chelating agent has the structure

; wherein

R represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, aryloxy, a heterocyclic group, an enzyme, antigen, or antibody;

R^1 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody;

R^2 represents carboxy, hydroxy, carbonyliminodiacetic acid, methyleneimino diacetic acid, hydrazinylylidene diacetic acid or the esters or salts of such acids; and
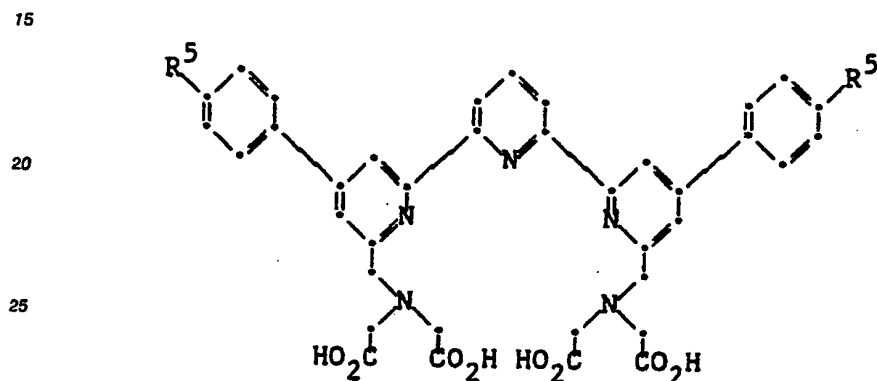
n is 1 to 4.

4. The invention of claim 1 or 2 wherein the chelating agent has the structure

; wherein

R¹ represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, substituted or unsubstituted aryl, a heterocyclic group, an enzyme, an antigen or an antibody; and

R² represents carboxy, hydroxy, carbonyliminodiacetic acid, methyleneimino diacetic acid, hydrazinylylidene diacetic acid or the esters or salts of such acids.

5. The invention of claim 3 wherein

R¹ represents hydrogen, phenyl, methoxyphenyl, tolyl, cyanophenyl, p-methylthiophenyl, methoxycarbonylphenyl, an enzyme, an antibody or an antigen or p-(5,5-diphenylimidazolidine-2,4-dion-3-yl)-methylphenyl; and

R² represents carboxy, hydroxy, carbonyliminodiacetic acid, methyleneimino diacetic acid, hydrazinylylidene diacetic acid or the salts of such acids.

6. The invention of claim 3 wherein the chelating agent has the structure

; wherein

R^4 represents $-H$, $-SCH_3$, $-OCH_3$, $-CN$, $-NO_2$, $-CO_2CH_3$;

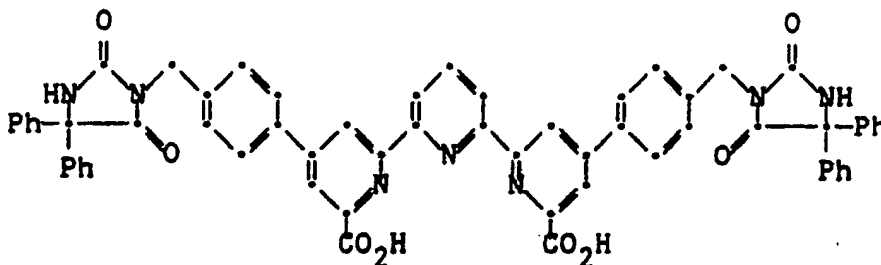
; wherein

R^5 represents $-CH_3$, $-NO_2$, or $-OCH_3$;

; wherein

R^{11} represents $-CH_3$, $-NO_2$, $-OCH_3$; or

34

7. The invention of claim 2, 3, 4, 5 or 6 wherein the physiologically active material is selected from antibodies, antigens, haptens, enzymes, enzyme substrates, metabolites, vitamins, hormones, hormone receptors, cell surface receptors and lectins.

8. The invention of any of Claims 1 - 6 wherein the mole ratio of Eu^{+3} to the chelating agent is from 1:1 to 2:1.

9. In a method for performing an immunoassay comprising the steps of

a) fluorescently labeling a ligand of interest, a derivative of said ligand or some other molecular entity, to produce a fluorescently labeled immuno logically binding ligand analog of said ligand of interest;

b) adding a known amount of said fluorescently labeled ligand analog and an unknown amount of ligand to be assayed to a receptor which is capable of binding with said fluorescently labeled ligand analog and also is capable of binding with said ligand to be assayed; and

c) determining the unknown amount of said ligand to be assayed by measuring the fluorescence of said fluorescently labeled ligand analog bound to said receptor or by measuring the fluorescence of said fluorescently labeled ligand analog remaining unbound,
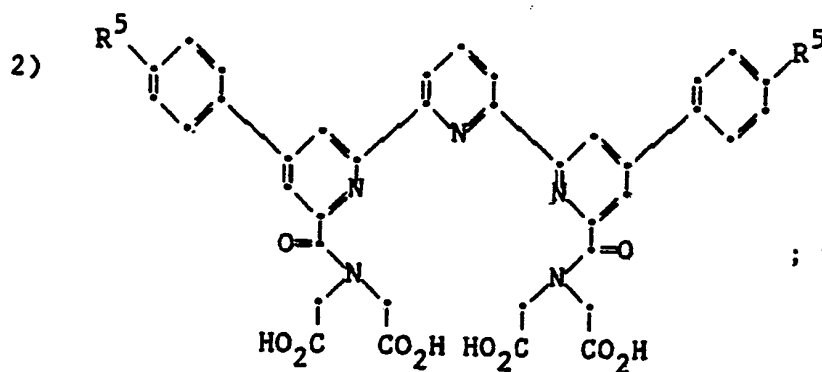
the improvement wherein the labeled ligand analog comprises ligand adsorbed or covalently bound to a fluorescent label comprising a fluorescent chelate of a lanthanide metal and a chelating agent as defined in Claim 1

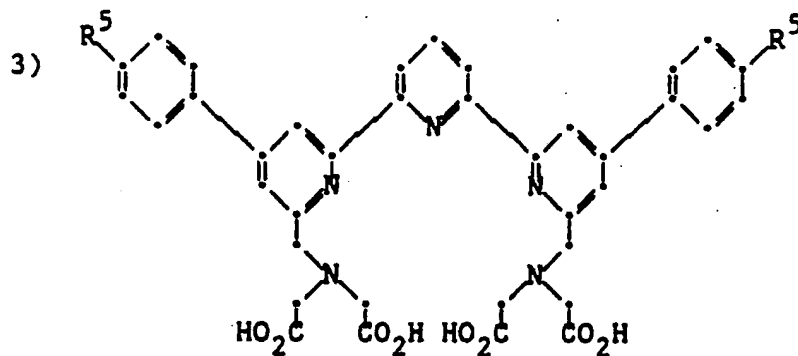10. A compound having one of the following structures

1)

; wherein

R^4 represents $-H$, $-SCH_3$, $-OCH_3$, $-CN$, $-NO_2$, $-CO_2CH_3$;

2)

; wherein

R^5 represents $-NO_2$, $-OCH_3$;

3) ; wherein

R^5 represents $-CH_3$, $-NO_2$ or $-OCH_3$.

4) ; wherein

R^9 represents $-H$, $-OCH_3$, $-NO_2$;

5)

CH_3O ... OCH_3

$(CH_3)_3CO_2C$ $CO_2C(CH_3)_3$

$CO_2C(CH_3)_3$ $CO_2C(CH_3)_3$

;

6)

Ph Ph

Ph Ph

CO_2H CO_2H

;

7)

R^7

; wherein

HO_2C CO_2H HO_2C CO_2H

R^7 represents $-H$, $-OCH_3$, $-NO_2$; and

8)

$$R^8$$

$$CO_2H \qquad CO_2H$$

; wherein

R^8 represents H, $-OCH_3$, $-NO_2$.